REDOX INDICATOR PATTERNS IN RELATION TO ECHINODERM EXOGASTRULATION. II. REDUCTION PATTERNS

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Continued use of redox indicators on echinoderm material during the last six years has brought to light certain characteristics of the patterns of intracellular oxidation and reduction of the indicators, particularly in their relation to exogastrulation, and has made it desirable to call attention again to certain features of these patterns. Some of these were not known to be present, and the physiological significance of certain others was still uncertain at the time of early studies of indicator patterns. Intracellular oxidation patterns have already been considered in a preceding paper (Child, 1953c), with suggestions concerning their significance in exogastrulation. The present paper is concerned with reduction patterns.

The first studies of redox indicator patterns in echinoderms also described only reduction patterns; these became visible after staining by certain redox dyes only when external oxygen was decreased to a certain critical level. The dyes became hydrogen acceptors and in the case of methylene blue and various other dyes merely became colorless, or with diazine green (Janus green) reduction to the red diethyl safranine occurred first and might be followed by further reduction to colorless, with return to red after oxygen increase (Child, 1936a, 1936b). In these papers presence of distinct regional differentials in rapidity of reduction was demonstrated. These constituted gradient patterns of reduction obviously correlated in some way with the physiological axes and with the course of morphogenesis. At that time nothing was known concerning intracellular oxidation patterns of the indicators; consequently the physiological significance of certain features of the reduction patterns was not clearly recognized.

As a background for the recent studies of reduction pattern, it seems necessary to call attention briefly to some of the more important results of the earlier papers. The material consisted of *Strongylocentrotus purpuratus, S. franciscanus, Dendraster excentricus,* all echinoids, and the asteroid starfish, *Patiria miniata.* In normal development (*i.e.*, the course or courses of development under as nearly as possible natural conditions and without experimental modification) the egg, cleavage stages, and the earlier blastulae showed a reduction gradient decreasing basipetally from the apical region without any visible change in this pattern at the time of formation of the micromeres in the echinoids. In *Patiria* there are no micromeres; also the basipetal differential in rate of reduction seemed to be somewhat greater in the starfish than in the echinoids. It was further noted that when the cells of the cell wall of blastulae and early gastrulae were stained throughout, reduction progressed from the blastocoelar surface outward, and during and after immigration of primary mesenchyme cells in echinoids they reduced more rapidly than any other cells. The decrease in rate of reduction from the blastocoel outward was regarded as resulting from lower oxygen content in the blastocoel than in the external fluid and was believed to be rather an incident of development than of any

real significance. The arrows used to indicate directions of decrease in rate of reduction were intended to indicate primarily differentials in the polar reduction gradients and other regional gradients of later development. In most figures the arrows indicate reduction beginning on the blastocoelar surface of the cell-wall, but in their further course they were often drawn entirely outside the body. With approach of gastrulation a second reduction gradient appeared in the basal region with decrease acropetally, and variation in extent toward the apical region with different experimental conditions.[1] In somewhat later studies a similar reduction pattern of intracellular indophenol was observed in *Dendraster* and *Patiria* (Child, 1941b, 1944).

In all these earlier papers attention was repeatedly called to the possible significance of this change in the intracellular reduction pattern with approach of gastrulation, as suggesting a change of some sort in the oxidation-reduction mechanism, apparently associated with activation of primary mesenchyme and prospective entoderm in echinoids and of prospective entoderm in the starfish, perhaps a step in differentiation of the basal region. It is accompanied or almost immediately followed by immigration of the mesenchyme in echinoids and by entodermal invagination in both echinoids and starfish.[2] In the early papers on reduction patterns it was further noted that in the entogastrula the reduction differential in the entodermal cell wall underwent a reversal in direction, at least in the apical entoderm, with reduction no longer progressing from the blastocoelar surface but from the archenteric cavity.[3] This change was regarded as probably resulting from lower oxygen content in the cavity than elsewhere. The apical archenteric region attains high developmental activity and high susceptibility to inhibiting agents during these stages. Adequate supply of oxygen through the blastopore (anus) appears improbable and fluid in the cavity apparently moves toward, rather than from the anus. Oxygen diffusing inward from the exterior must now pass, not only through the ectoderm, but through the entodermal cell-wall to reach the archenteric cavity. With external oxygen decrease by a reducing agent, and with the oxygen uptake of the ectodermal and entodermal cell-walls, it appears highly probable that oxygen content in the blastocoel will become lower than elsewhere, as the course of reduction indicates.

In the evaginated entoderm of the exogastrula no such reversal in direction in the entodermal cell-wall was found (Child, 1936b, p. 484). If the preceding interpretation of the reversal is correct, no reversal is to be expected. The internal entodermal cavity in the exogastrula is still a part of the blastocoel and diffusion of oxygen is merely through the entodermal cell-wall. With external oxygen decrease, the oxygen uptakes of the entoderm cells and of mesenchyme in the echinoids and dissociated entoderm cells in the starfish, which are evidently not dead in most cases, all contribute to decrease oxygen content in the blastocoel below that elsewhere and so to determine entodermal reduction in the exogastrula decreasing from the blastocoel outward. If the exogastrular ectoderm is thick enough to show

[1] The basipetal reduction gradient is indicated in Child, 1936a, Figs. 8–21, the later appearance of the acropetal reduction pattern in Figs. 22–26 and 29–31.

[2] Child, 1936a, p. 450; 1941a, p. 129; 1941b, p. 525, bottom; 1944, pp. 450–51. The reduction pattern in *Dendraster* was described only briefly without figures, 1941b. The new acropetal reduction of *Patiria* was indicated in Child, 1944, Figs. 11 and 13–20.

[3] Child, 1936a, Figs. 33 and 36–38.

a cell-wall gradient, reduction progresses from the blastocoel outward there, as in the entoderm.

The frequently repeated studies of redox indicator patterns of echinoderms in recent years confirm in general the earlier observations on the reduction patterns of normal development, and perhaps contribute something to the physiological analysis of these patterns. As regards exogastrulae, however, the earlier observations are less complete, and conditions determining the entodermal reduction pattern observed in almost all exogastrulae were not analyzed. In almost every one of thousands of exogastrulae reduction was found to begin at the ect-entodermal junction or in the entoderm near it. A very few elongated exogastrulae of *Dendraster* were found with entodermal reduction decreasing from the tip (Child, 1936b, Figs. 21 and 22) and later (Child, 1941b, p. 527) it was noted that in some elongated *Dendraster* exogastrulae entodermal reduction progressed from the tip, but little attempt to account either for the usual course of entodermal reduction from the ect-entodermal junction or the few cases of reduction from the entodermal tip was made. The rapid development, and often the great elongation of the entoderm in exogastrulae, suggest that the evaginating, like the invaginating, entoderm develops with activity decreasing from the tip. It was suggested in the early paper that this gradient might have been reversed in direction by the much greater susceptibility of the tip to the exogastrulating agent, but this suggestion was not fully discussed. It appears from recent studies that a reversal of this sort may occur in some of the more extreme degrees of exogastrulation, though by no means in all exogastrulae.

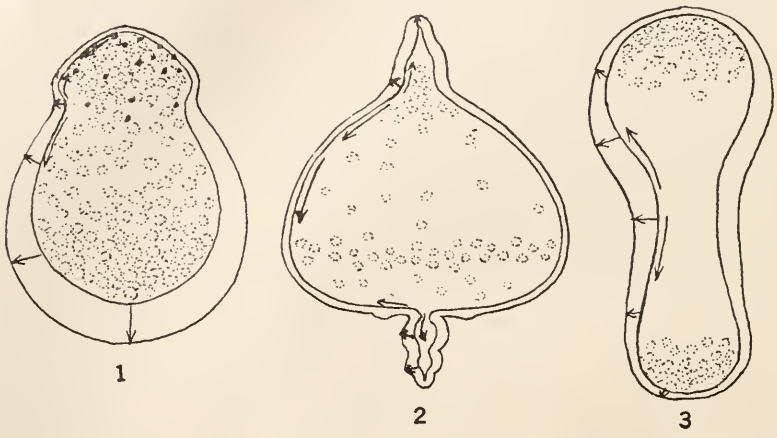
When the early studies were made it was not clearly recognized that the conditions under which the reduction pattern becomes visible represented, or might represent, differentially inhibiting factors in addition to the agent used for exogastrulation. If the tip of the evaginated entoderm is the most active and most susceptible region, the external oxygen decrease necessary for intracellular reduction may also be a highly important factor in decreasing or obliterating, or perhaps even reversing, the direction of the oxidation gradient. Lack of oxygen has been found to be a differential inhibitor with the same relation to oxidation gradient pattern as other differentially inhibiting agents.

Staining by oxidized dye, particularly by the relatively toxic diazine green, used very largely in the earlier study, is also a differential inhibitor with the same relation to gradient pattern as others. If staining is too long continued, reduction in the more susceptible region or regions is retarded. If the entodermal tip is the most susceptible region of the evaginated entoderm it will be most retarded in reduction. In the earlier study of reduction the oxygen decrease necessary for reduction was brought about gradually by the oxygen uptake of a number of animals sealed in a small volume of water or sometimes in dilute dye solution. Consequently oxygen decrease occurred gradually and the critical level for reduction was attained after a variable length of time. During this time the animals remained stained by oxidized dye.

And finally, it is possible that the physiological age of the exogastrula may sometimes be a factor concerned to some degree in determining the reduction pattern. In normal plutei and starfish larvae the oxidase gradient patterns gradually decrease with the progress of starvation of the larvae and may almost completely disappear while the larvae are still motile. The exogastrula is not a

stage in the progress of development. It represents the end of that form of development when it has attained its final stages. Exogastrulae may live for days after growth and elongation of the evaginated entoderm have ceased, but it seems probable that they usually, if not always, die from other conditions than starvation. The entodermal oxidase gradient evidently persists in exogastrulae for a considerable time (Child, 1953c); it sometimes seems to become less evident in older exogastrulae, but comparison of the degree of differential in this gradient or in the reduction gradient in different individuals is of no real value.

With continued exposure to moderate degrees of differential inhibition by the exogastrulating agent, there is the possibility of development of differential tolerance to the agent. With return of material to water, even after relatively extreme degrees of inhibition there is often a very considerable degree of differential recovery in definite relation to the gradient pattern, and a continuation of exogastrular



FIGURES 1–3. Slightly modified from figures of the early study of reduction pattern for greater clarity as regards gradient pattern: Figure 1, *Dendraster,* six days in LiCl *M*/50; Figure 2, *Strongylocentrotus,* extreme crowding in water; Figure 3, *Patiria,* 60 hours in LiCl *M*/30. Further data in text.

modification. Differences in susceptibility in eggs of the same or of different lots and the difficulty of exposing different individuals, even in the same container, to conditions that are really similar, emphasize the desirability of covering similar experimental ground repeatedly with different lots of material.

At the time of the first study of reduction pattern nothing was known concerning the indicator oxidation or oxidase pattern. The reduction pattern must now be considered in relation to what has been learned concerning the oxidase pattern. The oxidase gradient of the cell-wall decreasing from the blastocoelar surface in blastulae and early gastrulae of normal development (Child, 1953c) suggests that under natural conditions oxygen content in the blastocoel may not differ greatly from that outside but may become much less than outside after external oxygen decrease, in consequence of oxygen uptake by cells of the wall and mesenchyme and dissociated cells in the blastocoel. The purpose of this discussion is merely to call attention to various factors which are or may be con-

cerned in determining reduction patterns; few suggestions concerning the roles of particular factors in individuals are possible.

Figures 1–3 are exogastrulae of *Dendraster, S. purpuratus* and *Patiria* from the early paper, but with positions of the arrows indicating directions of decrease in rates of reduction altered in order to show more exactly the patterns described in the text of that paper. In Figure 1 (6 days in LiCl *M*/50 from 2-cell stage) the ectoderm still shows a very slight polar gradient. In Figure 2 (extreme crowding in water) there is evidently entodermization of the apical ectoderm with elongation of the entodermized region outward, as in basal exogastrulation (Child, 1948). In both the basal and the apical exogastrulation reduction progresses from the ect-entodermal junction and in the ectoderm the polar gradient is still present, except basally. In Figure 3 (60 hours in LiCl *M*/30 from 2-cell stage) reduction progresses from a region of the entoderm, perhaps entodermized ectoderm, nearer the ect-entodermal junction than the entodermal tip. In all cases reduction in the cell-wall progresses from the blastocoel outward. These were the reduction patterns observed in thousands of exogastrulae of all four echinoderms.

Material and Methods

The three echinoderms chiefly used in the early study of reduction and in the preceding paper on oxidation pattern, *Dendraster excentricus, S. purpuratus* and *Patiria miniata,* were again used in this further study of reduction patterns.[4] The dyes used were chiefly diazine green and in some cases methylene blue. Diazine green, with the two steps in reduction and with color change, first from the blue-green of the oxidized dye to the red diethyl safranine, and second, the further reduction to colorless, has been more useful than other dyes which merely lose color on reduction. Intracellular reoxidation of colorless reduced diazine green to the red diethyl safranine is possible, but further reoxidation to the blue green, fully oxidized dye does not usually occur in echinoderm material, though it has been observed in some other organisms. Diazine green is more toxic than many other "vital" dyes and has been used in concentrations of 1/100,000 and 1/50,000, and usually only with staining periods from 5–15 minutes.

Patterns of intracellular indophenol reduction with loss of color are similar to the dye reduction patterns. As repeatedly described in earlier papers, intracellular indophenol reaction (the Nadi reaction) results from oxidation of the reagents, para-aminodimethyl aniline (dimethylparaphenylene diamine) and α-naphthol, catalyzed by an oxidase, often regarded as cytochrome oxidase. Both of the indophenol reagents are toxic but with use in very low concentrations both the intracellular reaction with deep blue color and reduction to colorless are possible in apparently uninjured embryos and larvae and in motile stages still moving. Also alkali is not required to dissolve the naphthol. Use of this indicator is more fully described in earlier papers (*e.g.,* Child, 1944, 1953c and various other papers).

[4] The kindness of the Director and staff of the Hopkins Marine Station in providing material, facilities for work, and in some cases for transportation of material to Palo Alto, and of Dr. Olin Rulon for sharing *Dendraster* material and transporting it to Palo Alto is again gratefully acknowledged.

Although certain organisms or certain regions or organs can reduce intracellular methylene blue and low intracellular concentrations of diazine green and indophenol without external oxygen decrease, intracellular reduction of these and various other redox indicators is not generally characteristic of development or life under natural conditions, but occurs only after oxygen in the external environment and in the tissues has undergone decrease to a critical concentration. The indicator then becomes a hydrogen acceptor and is reduced. This reduction is considered to be catalyzed by one or more dehydrogenases. Obviously this reduction of the indicator represents oxidation of some intracellular substrate by loss of hydrogen to the indicator and becomes the characteristic reaction with sufficient oxygen decrease.

Decrease of available oxygen can be brought about in various ways. In some of the earliest studies of intracellular reduction of indicators highly toxic reducing agents were used, *e.g.*, sodium hyposulphite and hydrochloric acid, also another, rongalite, containing formalin. These required extreme caution in use and in certain cases their use led to errors as regards regional differentials in rates of reduction. Regions most susceptible to the toxic effects were injured, so that reduction in them was delayed or did not occur, though when they were not greatly injured reduction was more rapid in them than in any other parts (Child, 1941a, pp. 90–92 and footnote). In the earliest studies of indicator reduction in echinoderms these toxic reducing agents were not used. After staining by oxidized dyes a number of embryos or larvae were sealed in a small volume of water or, in some cases, dilute dye solution and oxygen decrease resulted from oxygen uptake of the living material. With this procedure the length of time before reduction varied with number of individuals and volume of fluid. Intracellular reoxidation occurred rapidly on opening the sealed preparation and reduction and reoxidation could often be repeated several times before the intracellular concentration of oxidized indicator became toxic. Some years later this method was used for intracellular indophenol reduction in *Dendraster* (Child, 1941b).

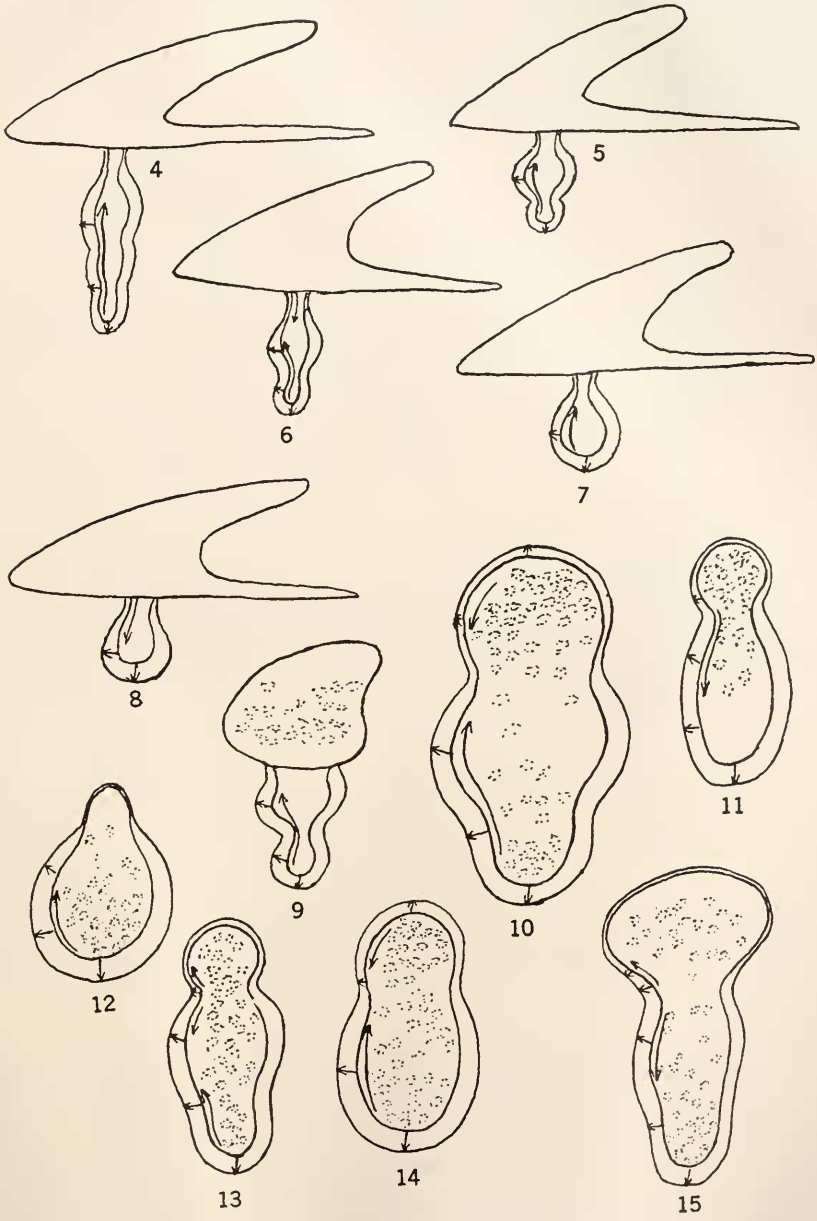
In recent indicator studies, however, a much more satisfactory reducing agent, sodium hydrosulphite ($NaHSO_2$ or $Na_2H_2S_2O_4$), has been used. A fraction of a milligram is sufficient to bring about reduction in echinoderm developmental stages in one ml. of water. The chief difficulty is to keep the quantity used small enough so that reduction will not be too rapid for observation of regional differentials. This agent is not appreciably toxic in concentrations much higher than those required for reduction, though in sealed preparations animals finally die from lack of oxygen. Hydrosulphite has been used for reduction in all cases considered in this paper and in thousands of other individuals.

Figures are essentially optical sections along the polar axes. They do not indicate actual differences in size in the different species. Arrows, drawn only for the left side, though the two sides are similar, point in the direction of decrease in rate of reduction. Mesenchyme and dissociated entoderm cells in the blastocoels are indicated in dotted outlines or areas.

Reduction Patterns of Dendraster and Strongylocentrotus Exogastrulae

Exogastrular reduction patterns and their variations are similar in these two echinoids. A highly effective method for producing exogastrulation in these forms is exposure to low temperature during early development with later de-

velopment at a much higher temperature. Figures 4–8 are from a lot kept at 10° C. for 29 hours from the 2–4 cell stage and later at 22°–24° C. Neither change of temperature was sudden. At the low temperature development did not usually

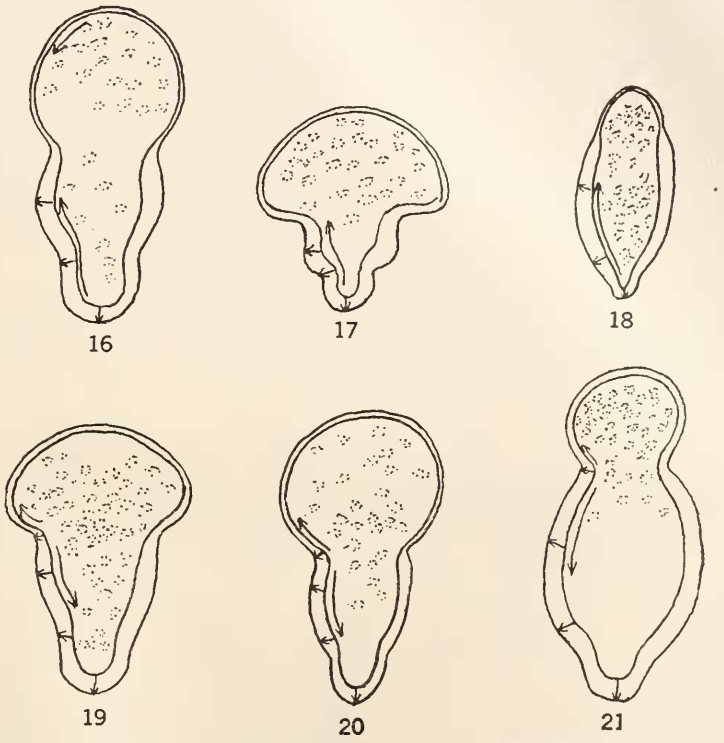


FIGURES 4–15. Reduction patterns in *Dendraster* exogastrulae: Figures 4–8, exogastrulae resulting from temperature change; Figure 9, 2 days in sodium azide *M*/600; Figures 10–15, 2 days in water after 28 hours in LiCl *M*/50. Further data in text.

progress beyond blastula stages and if these were left too long at the low temperature death occurred. Figures 4–8 represent exogastrulae with high degrees of differential recovery at the higher temperature. Ectoderm and mesenchyme attain complete pluteus development and in Figures 4–6 there is more or less development of three entodermal segments. More extreme degrees of exogastrulation occur with slightly longer exposure to low temperature. In Figures 4–6 entodermal reduction progresses from the tip, though in Figure 6 the thin-walled region adjoining the ectoderm reduces from the ectoderm. In Figure 7, with less entodermal development, reduction progresses from the tip, but in Figure 8, with very similar degree of entodermal development, entodermal reduction progresses from the ectoderm. Reduction in the entodermal cell-wall progresses from the blastocoel outward. Ectodermal reduction pattern is like that of the normal pluteus; tips of the oral lobe and of the arms are the high ends of reduction gradients. Figure 9 with ectodermal development stopped in a prepluteus stage, and entodermal reduction progressing from the tip was exposed to sodium azide only after it attained the blastula stage (azide *M*/600, 2 days). With this relatively later exposure to azide only a small number of exogastrulae appeared; inhibited entogastrulae developed in an estimated 95 per cent of the lot, and the few exogastrulae were not extremely inhibited by azide. Figures 10–15 represent exogastrulae two days in water after 28 hours from the 2–4 cell stage in LiCl *M*/50. All are from a single container and serve as examples of the variations in form and development under more or less similar conditions. In Figures 10–12 entodermal polar reduction progresses from the tip and in Figures 10 and 11 a slight ectodermal polar gradient, decreasing basipetally, still persists. Figure 12 is particularly interesting; evidently it was greatly inhibited by LiCl, apparently with entodermization of much of the ectoderm, but reduction progressing from the entodermal tip suggests that after return to water the entoderm underwent a high degree of differential recovery, with the tip becoming the region of most rapid reduction. Figure 13 is also of interest, as suggesting some degree of recovery of the entoderm at and near the tip, though not sufficient to prevent occurrence of reduction in both directions at the ect-entodermal junction. In Figures 14 and 15 reduction progresses in both or only in one direction from the ect-entodermal junction, the usual course of reduction in the early study of exogastrulation. Similar varieties of form and course of reduction occur very generally in a single container, except with extreme degrees of inhibition by the exogastrulating agent or other conditions. In all cases in which the entodermal cell-wall is thick enough to show the cell-wall gradient clearly, reduction progresses from the blastocoelar surface outward.

Exogastrulae of *Strongylocentrotus* differ so little from those of *Dendraster* that they require only brief attention. Figures 16 and 17 are forms two days in water after two days in azide *M*/800 from 2–4 cell stages. There was evidently considerable differential recovery after return to water, with further development of entoderm. Reduction progresses from the entodermal tip and in Figure 16 a slight polar gradient is present in the ectoderm. Figure 18, from a lot two days in LiCl *M*/50 from the 2-cell stage with dilution of the solution to approximately half water after that period, is much like Figure 12 of *Dendraster*. Here also there is probably entodermization of ectoderm and differential recovery of entoderm

with reduction progressing from the entodermal tip. Many other exogastrulae in this lot were very similar. In Figure 19, after three days in LiCl $M/40$ without return to water, reduction is from the ect-entodermal junction. Figure 20, three days in LiCl $M/60$ without return to water, also showed reduction from the ect-entodermal junction. Figure 21, from a lot one day in LiCl $M/30$, followed by two days in water, shows reduction progressing from the ect-entodermal junction, like most others in the lot, but in a few individuals reduction progressed from the entodermal tip. Perhaps the *Strongylocentrotus* exogastrulae, not merely



FIGURES 16–21. Reduction patterns in *Strongylocentrotus* exogastrulae: Figures 16 and 17, 2 days in water after 2 days in sodium azide $M/800$; Figure 18, 2 days in LiCl $M/50$ followed by dilution to approximately $M/100$; Figure 19, 2 days in LiCl $M/40$; Figure 20, 3 days in LiCl $M/60$; Figure 21, 2 days in water after one day in LiCl $M/30$. Further data in text.

those of the figures, but many hundreds of exogastrulae in many lots, suggest that recovery of the evaginated entoderm, so that reduction progresses from its tip occurs less frequently in this echinoid than in *Dendraster*. This difference is perhaps to be expected, as *Strongylocentrotus* is in general somewhat more susceptible to inhibiting conditions than *Dendraster*. In both forms reduction in the cell-wall progressed from the blastocoelar surface outward in all cases in which the cell-wall was not so thin that the gradient was uncertain.

It is a point of incidental interest that in these echinoid exogastrulae there are usually free cells in the blastocoel; immigration of mesenchyme or of some part of

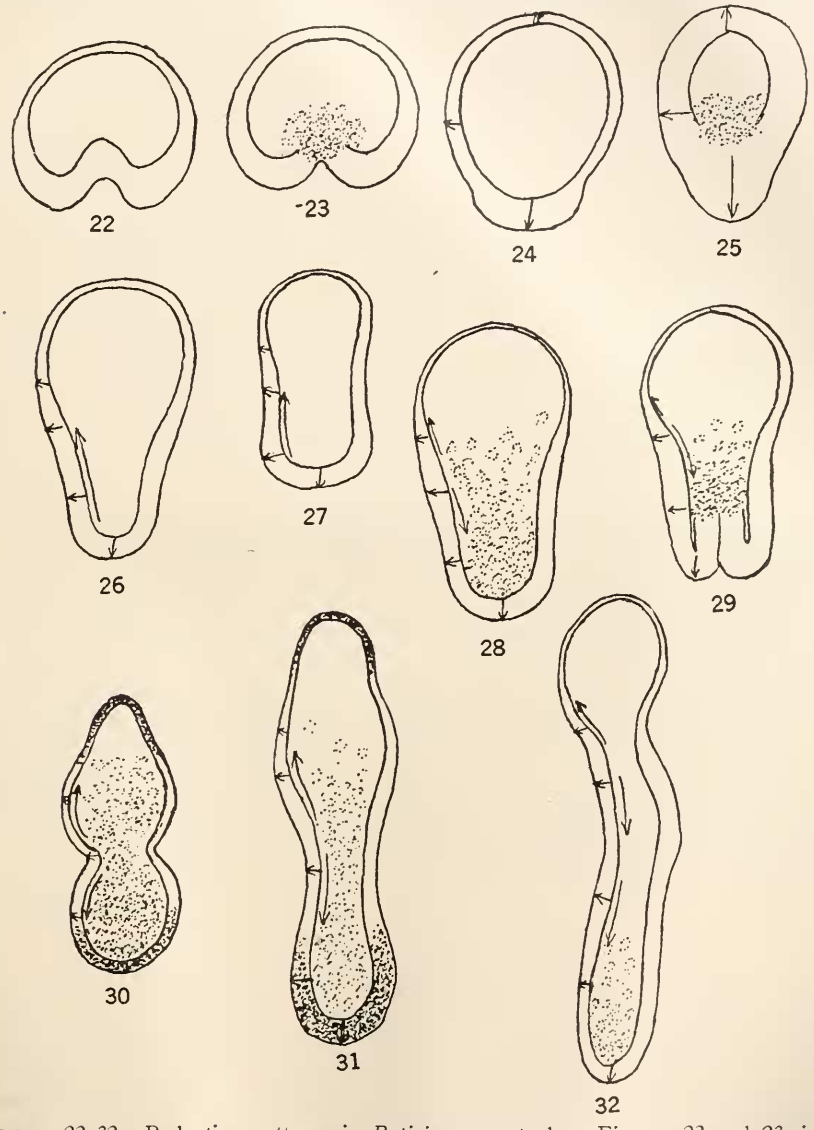
it often occurs before actual evagination of entoderm. Not infrequently most of the mesenchyme cells reach the ectodermal region, but, except in the lesser degrees of exogastrulation such as Figures 4–8 of *Dendraster*, the ectodermal factors localizing mesenchyme are obliterated, and, in Figure 9, almost obliterated. In those exogastrulae skeleton does not develop, or at most a few irregularly localized spicules or rods appear. In addition to mesenchyme cells in the blastocoel, cells may dissociate internally from the entoderm; these are usually still capable of reduction and oxidation of indicators. These dissociated cells in the blastocoel reduce earlier than other parts of the exogastrulae, and, as pointed out above, doubtless play some part in decreasing oxygen content in the blastocoel by their own oxygen uptake, *i.e.*, as external oxygen decrease occurs these cells probably determine their own rapid reduction and may also be factors in determining the cell-wall gradient decreasing from the blastocoel outward. All figures of echinoid exogastrulae were drawn from individuals in which direction of the cell-wall gradient was clearly distinguishable. In some exogastrulae the entoderms may become so thin that direction of the cell-wall gradient becomes difficult or impossible to determine. Occasionally it was noted at the time of observation that reduction seemed to progress from the external entodermal surface inward. With rapid and extreme oxygen decrease, this is of course possible, but it seems quite beyond question that the characteristic cell-wall reduction gradient decreases from the blastocoel outward in the evaginated entoderm and also in the ectoderm, if that is not too thin to show a distinct cell-wall gradient.

Reduction Patterns in Exogastrulae of Patiria

With exposure to exogastrulating agents, beginning in the earlier blastula stages, development of differentially inhibited entogastrulae may precede exogastrulation. For example, Figures 22 and 23 are from a lot in which every individual of several samples, including large numbers, was an inhibited entogastrula after 15 hours in LiCl *M*/30 from early blastula stages. In many of these, entodermal dissociation was already occurring. After 48 hours in LiCl every individual of numerous samples was an exogastrula and in most of them the invaginated part of the entoderm was dissociating or dissociated. Figure 24 is an exogastrula with its development stopped in early stages during three days in a high concentration of LiCl (*M*/7.5). Only the cell-wall gradient, decreasing from the blastocoel outward, is distinguishable. Figure 25 is also stopped in an early stage of exogastrulation by three days in azide *M*/250. Entodermal dissociation is beginning internally and here also only the cell-wall gradients are distinguishable.

Figures 26 and 27, two days in LiCl *M*/30 from early blastula stages, are cases of polar entodermal reduction progressing from the tip and without entodermal dissociation. This pattern of reduction has not been observed as frequently in *Patiria* as in the echinoids; in those cases in which it has been observed, no evidence of entodermal dissociation has appeared. In Figures 28–32 polar entodermal reduction progresses in both directions from an entodermal region near the ect-entodermal junction and all show more or less entodermal dissociation. Figure 28, 2 days in LiCl *M*/30 from the early blastula, may have been originally an entexogastrula; if it was, the invaginated part of the entoderm has dissociated internally and the remaining entoderm cells have come together and are intact.

Figure 29, with similar LiCl exposure, is an entexogastrula with the invaginated part of the entoderm in process of dissociation. In Figures 30 and 31, also with the same LiCl exposure, entodermal dissociation occurred earlier, perhaps during an entogastrula stage, and the remaining entoderm has healed. Figure 32, three days in LiCl *M*/30, is an example of the great length sometimes attained by the



FIGURES 22–32. Reduction patterns in *Patiria* exogastrulae: Figures 22 and 23, inhibited entogastrulae after 15 hours in LiCl *M*/30, after 48 hours in LiCl were exogastrulae; Figure 24, exogastrula stopped in early stage after 3 days in LiCl *M*/7.5; Figure 25, stopped in early exogastrulation after 3 days in sodium azide *M*/250; Figures 26–31, 2 days in LiCl *M*/30 from early blastula stages; Figure 32, 3 days in LiCl *M*/30. Further data in text.

evaginated entoderm when little or no dissociation occurs. These figures suggest that the most active and therefore the most susceptible entodermal region often undergoes dissociation; other less susceptible regions remain intact and may apparently develop some degree of tolerance to this concentration of LiCl and undergo further elongation (Figs. 31 and 32).

Figures 30 and 31 are intended to indicate another interesting characteristic of starfish exogastrulae. In the course of observations of reduction of oxidized diazine green it was found, first by accident and later confirmed by many cases, that when staining by this dye was continued somewhat longer than the usual 5–15 minutes with 1/100,000 or 1/50,000, reduction occurred as the arrows in these figures and in Figures 28 and 29 indicate. Reduction to the red diethyl safranine occurred first; reduction to colorless followed in the dissociated cells but a variable region of the ectoderm and of the entodermal tip remained red and did not reduce to colorless at any time. In Figures 30 and 31 this is indicated by the deep shading of the apical region of the ectoderm and of the entodermal tip. In some cases in which lots were left in oxidized diazine green for half an hour or somewhat more, these two regions remained blue-green, *i.e.*, did not reduce at all. These cases are regarded as indicating differential injury of these regions, the most susceptible of the individual, while the less susceptible are still able to reduce the dye, even to colorless. In use of diazine green on other organisms and even with other less toxic dyes it has been found that with over-staining by oxidized dyes reduction is retarded or may not occur at all. Indophenol reduction is retarded similarly if the intracellular concentration of indophenol becomes sufficiently high.

Although the length of the evaginated entoderm in the starfish exogastrulae varies greatly, further differentiation of the entoderm with development of two or three segments has been observed only in nine individuals among the thousands of exogastrulae observed. In the starfish, as in other echinoderms, degrees of exogastrulation and indicator patterns are dependent on experimental procedures, temperatures and susceptibilities of individuals and different lots of eggs. It is of course possible that with different exposure periods to exogastrulating agents or other differences in experimental conditions frequencies of the different reduction patterns may differ. However, the present paper is primarily concerned with occurrence, rather than with frequencies of the different patterns.

There is no primary mesenchyme in the starfish. The dissociated cells in the blastocoels of most starfish exogastrulae are cells dissociated from the entoderm, but usually still capable of reducing and reoxidizing the indicators. They unquestionably contribute to the low oxygen content in the blastocoel and, like the mesenchyme of the echinoids, usually reduce before other parts. Apparently the entoderm cells which dissociate into the blastocoel represent the most susceptible entodermal region; when there is no dissociation, reduction progressing from the entodermal tip appears to be more frequent than in cases of dissociation of this region. The progress of reduction in the entodermal cell-wall from the blastocoel outward is even more distinct in the starfish than in the echinoids; the larger size of starfish stages and the greater thickness of the evaginated entoderm in most of the exogastrulae account for this difference.

Entodermal Reduction in Normal Development Without External Oxygen Decrease

Although this section concerns certain observations on normal development, rather than on exogastrulae, it is included here as an example of determination by metabolic activity of decrease in oxygen content in internal cavities below that in external environment, and therefore as bearing on certain questions of reduction pattern.

It was recently observed that in normal plutei and somewhat earlier stages of *Dendraster* after 10–15 minutes in oxidized diazine green the mesenchyme cells and the archenteron became distinctly red in well-aerated water, though the ectoderm remained completely oxidized. Indophenol reduction of the archenteron also occurs without external oxygen decrease, though this reduction consists merely in loss of color and is less striking, and must also be observed through the more or less deep blue ectoderm. In the midgut with thicker cell-wall than other parts of the entoderm reduction appears to progress from the internal cavity toward the blastocoelar surface. As noted above, oxygen diffusing inward must now pass through two cell-walls to reach this cavity and muscular contraction of the gut begins to occur at about this time. To what extent oxygen reaches the archenteric cavity from the mouth is not known at present; evidently it is not sufficient in amount to prevent low oxygen content and early reduction in the cavity. Apparently the hindgut also undergoes early reduction from the inside outward.

In the later stages of normal larval development of *Patiria* entodermal reduction of diazine green to the red diethyl safranine occurs more rapidly and to a greater degree than in *Dendraster* after a few minutes in the oxidized dye and in well-aerated water, while ectoderm remains completely oxidized. Indophenol reduction also occurs under the same conditions, though if the ectodermal reaction becomes deep in color it may be difficult to observe. Here, even more distinctly than in *Dendraster*, reduction progresses from the entodermal cavity toward the blastocoel, at least in the midgut and apparently in the hindgut. The foregut becomes thin-walled as it enlarges, and direction of reduction is less clearly distinguishable there. Oxygen entering through the mouth is evidently not sufficient in amount to prevent this early entodermal reduction. Probably the sea urchin will also show early entodermal reduction in later stages of normal larval development without external oxygen decrease, but this question must await another breeding season.

Discussion and Conclusions

As regards the pattern of indicator reduction, it must again be emphasized that intracellular reduction of the indicators represents an intracellular oxidation in the living tissues. With oxygen decrease to a critical point the indicator becomes a hydrogen acceptor with catalysis of the reaction by dehydrogenase. Some substrate in the cells loses a hydrogen to the indicator. The substrate concerned in this dehydrogenase oxidation is undoubtedly different from that involved in the oxidase or oxidation patterns. In other words, the pattern of indicator reduction is actually the pattern of certain intracellular oxidations, a component of the metabolism of the living cells.

In the echinoderms thus far investigated by means of redox indicators the patterns of intracellular oxidation, catalyzed by an oxidase or by oxidases, and the patterns of indicator reduction, catalyzed by one or more dehydrogenases, are the same, as regards regional differentials, in early development under natural conditions to stages just preceding gastrulation and in early stages of entodermal invagination. As those stages are attained, the primary mesenchyme cells of the echinoids and the prospective entoderm, previously the least active region, evidently undergo a considerable activation, apparently involving rapid growth of the entoderm. With this activation a new reduction gradient decreasing from the basal region, *i.e.*, opposed in direction to the reduction gradient of earlier stages and also to the oxidase or oxidation gradient, appears. The oxidase gradient remains unaltered and still decreases from the apical region basipetally over the entire individual. The new acropetal reduction gradient varies in length according to degree and duration of oxygen decrease. It may extend into the ectoderm. Attention was repeatedly called to the appearance of this new reduction pattern, and it was suggested that it indicated a change in physiological condition, decreasing from the basal region acropetally, and perhaps representing a step in differentiation.[5] In later gastrula and larval stages oxidation and reduction patterns again become similar as regards regional differentials and show the same relation to morphogenesis in normal development. In exogastrulae differences in relations of oxidase and reduction patterns appear. The polar patterns of both indicator oxidation and reduction may be decreased or entirely obliterated. The polar oxidation pattern in the evaginated entoderm is still present with decrease from the entodermal tip, though its differential may become slight when entodermal elongation is inhibited at an early stage (Child, 1953c). The reduction pattern of the evaginated entoderm may decrease from the tip or from the ect-entodermal junction or an entodermal region near it. In general, reduction from the entodermal tip, like that in entogastrulae, seems to occur more frequently, as might be expected, in the less extreme degrees of exogastrulation, though differences in susceptibility to inhibiting conditions differ so greatly in individuals and in different lots of eggs that it may also appear in more extreme forms. These variations in the entodermal reduction pattern indicate that the inhibiting conditions, noted above as necessary for exogastrulation, may obliterate, or perhaps reverse, the polar entodermal gradient. Under these conditions, the region at or near the ect-entodermal junction becomes the most active and most rapidly reducing region, not through increase in its own activity, but in consequence of inhibition of other regions.

In the starfish exogastrulae, and to a lesser degree in the echinoids, the dissociation into the blastocoel of cells from the entodermal tip apparently constitutes loss of the most active and most susceptible cells from the entodermal cell-wall. Even after dissociation, these cells, free in the blastocoel, may, and almost always do, reduce more rapidly than other cells, but they are no longer a part of the entodermal gradient pattern. After such dissociation, together with other condi-

[5] Child, 1936a; 1941a, pp. 133–143 and figures on these pages; 1941b; 1944. The writer is indebted to Dr. E. L. Tatum for the suggestion that the new reduction pattern might be associated with an increase in synthetic activity chiefly in the prospective entoderm, and that the two opposed gradient patterns may perhaps be regarded as indicating a competition of different intracellular substrates as regards oxidation, one catalyzed by oxidase with relatively high oxygen tension, the other catalyzed by dehydrogenase and favored by low oxygen tension.

tions tending to obliterate the entodermal polar gradient, reduction from the ect-entodermal region becomes increasingly probable. In the starfish reduction from the tip of the evaginated entoderm has usually been observed only when dissociation of entodermal cells did not occur. In the exogastrulae of *Dendraster* determined by change from low to high temperature reduction progressed from the entodermal tip with few exceptions in large numbers observed. Differential recovery after return to water following exposure to the inhibiting agent may also permit reactivation of the entodermal gradient and reduction from the tip.

The general polar and ventrodorsal gradients of the echinoderm embryo and larva are obviously different in character from the gradients between the surfaces of the cells which constitute the cell-walls of the embryo and larval stages. Polar and ventrodorsal gradients are differentials from cell to cell, involving the entire individual or extensive regions of it, though slight differentials may be present within the limits of single cells, *e.g.*, in cleavage stages (Child, 1953c). These general body-gradients are apparently determined in the ovary; at least this appears clearly to be the case as regards the polar gradient. However, this gradient can be decreased or even obliterated by differentially inhibiting conditions, and with differential recovery or with certain degrees of differential tolerance, the differential may become greater than in normal development, not only in echinoderms, but also in various other organisms. In a coelenterate early developmental stage it has been possible to determine experimentally a new polarity, and in reconstitution new polarities have been determined experimentally in many forms (Child, 1941a, Chapters X and XI). When and how the ventrodorsal gradient is determined still remains uncertain, though it may perhaps also be in the ovary in association with the growth of the gonad. It can be modified experimentally by the same conditions as the polar gradient and it can be experimentally reversed in direction, apparently by a differential inhibition (Pease, 1941, 1942a, 1942b). The cell-wall gradients are usually differentials between the two exposed surfaces of single cells, though in the thick entodermal masses of some exogastrulae and certain other modified forms the cell-wall is not a single cell-layer, and the cell-wall gradient becomes a multicellular differential. In the preceding paper it was pointed out that the presence in early normal development of the cell-wall gradient, decreasing from the blastocoel outward, did not support the earlier conclusion, based on reduction alone, that oxygen content in the blastocoel was normally less than externally (Child, 1936a). If this were the case, it seemed improbable that the oxidation gradient of the cell-wall could decrease from a region of lower, to one of higher, oxygen content. Moreover, the cell-wall oxidation gradient is not present from the beginning of development. Its appearance as a visible gradient, decreasing from the blastocoel outward, is associated with the appearance of the blastocoel. No visible indication of presence of this gradient has been observed in the 16-cell stage, but at 32 cells a slight cell-wall oxidation differential has been observed and later it becomes increasingly visible. It still remains a question as regards the conditions which determine the origin of this gradient with its high end toward the blastocoel in normal development. Further investigation is necessary to determine whether it is in any way correlated with position of the nucleus in the cell. Most figures of echinoderm larval development do not show the nuclei. There are, however, a few figures with nuclei in the

earliest studies of exogastrulae by Herbst,[6] but even these are not satisfactory. In some of them, at least some of the nuclei seem to be slightly nearer the blastocoelar, than the outer surface of the wall; in others no distinguishable difference with respect to the two surfaces of the wall appears. As regards the presence of this oxidation gradient there is no question. It has been observed in many hundreds of blastulae and early gastrulae, and its presence has been confirmed by others. It is possible to advance various hypotheses as regards conditions determining this gradient but at present they are little more than guesses.

The presence of this gradient in normal development by no means excludes the possibility that with external oxygen decrease oxygen content in the blastocoel may become much lower than outside in consequence of oxygen uptake by cells of the wall and cells which happen to be in the blastocoel, that the indicator reduction gradient may also decrease in the cell-wall from the blastocoel, and that immigrated mesenchyme and dissociated entoderm cells in the blastocoel may reduce most rapidly of all. Reduction from the internal cavity of the gut in later larval stages, entirely without external oxygen decrease, is further evidence along this line.

The cell-wall oxidation gradient of the entoderm undergoes reversal in direction with entodermal evagination; it was suggested in the preceding paper that this reversal is an essential factor in exogastrulation (Child, 1953c). In the exogastrular ectoderm there is no reversal of the oxidation gradient.

The indicator reduction gradient in the cell-wall of the evaginating or evaginated entoderm of exogastrulae undergoes no reversal and no definite changes. It becomes visible only after marked external oxygen decrease, and evidently in exogastrulae, as in normal development, results from more rapid oxygen decrease in the blastocoel than externally. The only difference from normal development is that in the evaginated entoderm the inner surface, in normal development, the outer surface, is the blastocoelar surface of the cells.

Whether an escape or partial escape from physiological dominance of a metabolically more active region of the developing echinoderm larva or an integrating factor or factors of some sort which may be concerned in what we call normal development, is concerned in exogastrulation, as suggested by J. W. MacArthur (1924), remains a question. Obviously the normal larva does not develop as an aggregation of independent parts. It is also obvious that the controlling or integrating factor or factors concerned in normal development are altered or, at least in part, absent in exogastrulae and other experimental developmental modifications. In the exogastrula the relation of ectoderm and entoderm has become different from that in the normal larva. In the elongated exogastrulae with great over-development of entoderm, and often more or less entodermization of ectoderm and dissociation of cells, the entoderm seems, to a greater or less extent, to have gained the upper hand. To that extent, apparently extreme in some exogastrulae, there appears to be a partial, or in some cases almost complete, entodermal independence. It develops as far as available material permits. Regional differential susceptibility to experimental differentially inhibiting factors is involved in this more or less extreme alteration or breakdown of physiological dominance or integrating and ordering factors concerned in determining a normal individual.

[6] Herbst, 1895, various figures, Plate IX; Figures 42 and 43, Plate X. 1896, Plate XXVI.

Summary

1. The paper consists primarily of a new investigation of intracellular reduction patterns of certain redox indicators in relation to exogastrulation, with the echinoids, *Strongylocentrotus purpuratus,* and *Dendraster excentricus,* and the asteroid, *Patiria miniata,* as material. Its purpose is: first, to record results of recent studies of these patterns, made with more adequate conditions for reduction than in earlier work; and second, to attempt somewhat further physiological analysis of the patterns and of their relation to oxidation patterns, than was undertaken in the earlier study.

2. Conditions which make the reduction patterns visible involve, not only the differentially inhibiting action of the exogastrulating agent, but also differentially inhibiting effects of oxygen decrease externally, and in some cases, of intracellular concentrations of oxidized dye or indophenol, and perhaps also the physiological age of the exogastrula. Usually the significance of these different factors for individual exogastrulae is not certainly distinguishable, but the differentially inhibiting effect of the exogastrulating agent is probably in general the most important.

3. In the less inhibited and less extreme forms of echinoid exogastrulae, in which ectoderm attains or approaches fully developed pluteus differentiation, the evaginated entoderm almost always reduces progressively from the tip toward the ectoderm, though occasional alterations of this pattern appear. In *Patiria* exogastrulae, dissociation of entoderm cells from the most susceptible and most inhibited entodermal tip and adjoining regions into the blastocoel occurs very frequently. Entodermal reduction progressing from the tip has been observed less frequently in *Patiria* than in the echinoids and thus far only when little or no dissociation from the entodermal tip occurs. In the echinoids entodermal dissociation may also increase the cells in the blastocoel far beyond the usual number of mesenchyme cells. In general, it appears evident that the larger the number of dissociated cells in the blastocoel, the less frequently does reduction progress from the entodermal tip.

4. When entodermal reduction does not progress from the tip, it begins at or near the ect-entodermal junction, or in *Patiria* in the entoderm near this junction, and progresses toward the entodermal tip and often acropetally for a short distance in the adjoining ectoderm. Under natural conditions, these regions are the least rapidly reducing regions of the entoderm after its activation preceding gastrulation, and of the adjoining ectoderm. In these exogastrulae they have become the regions of most rapid reduction, probably not by change in their own conditions, but by more extreme inhibition of other parts and obliteration or perhaps reversal in direction of their polar gradients.

5. In completely radial exogastrulae with rounded ectoderm lacking differentiation a slight polar reduction gradient may still be visible in the apical region, usually in cases of some degree of differential recovery after return to water, perhaps sometimes with development of differential tolerance to the exogastrulating agent, or a polar ectodermal reduction gradient may be completely absent. The ventrodorsal ectodermal gradient is completely obliterated by less extreme inhibition than the polar gradient.

6. Even if oxygen content in the blastocoel differs little or not at all from that in the external water, as the oxidase gradient of normal development seems to

indicate, it may become much lower in the blastocoel than outside, in consequence of oxygen uptake of cells of the cell-wall and of dissociated cells in the blastocoel with sufficient decrease of external oxygen tension. Under these conditions reduction must occur most rapidly in dissociated cells in the blastocoel, which are evidently not dead in most cases, and in the cell-wall reduction will progress from the blastocoelar surface outward in exogastrulae, as well as in normal development, and not only in entoderm, but also in ectoderm unless this has become so thin that a cell-wall gradient is not distinguishable. As might be expected, the cell-wall reduction gradient does not undergo reversal in exogastrulation, as does the oxidase gradient. The reduction gradient in the cell-wall is not directly related to exogastrulation.

7. In intracellular indicator reduction the indicator becomes a hydrogen acceptor and an intracellular substrate undergoes oxidation catalyzed by one or more dehydrogenases. Both intracellular oxidation of reduced redox indicators, catalyzed by oxidase, and intracellular reduction, catalyzed by dehydrogenase, are directly visible evidences of certain characteristics of oxidative metabolism, though different enzyme systems and undoubtedly different intracellular substrates are concerned in the two oxidative reaction systems.

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